derivative and nitrile separated essentially as described above under the procedure for *anti*-aldoximes.

In experiments (9), (10) and (11) of Table II, 1 g. of the *anti*-aldoxime was dissolved in 5 cc. of pyridine and 2 cc. of triethylamine or 2.5 cc. (2 equivalents) of tri-*n*-propylamine and the solution treated with 1 cc. of benzoyl chloride dissolved in 5 cc. of pyridine. The products were isolated essentially as described above.

Summary

1. The reaction of *anti*-aldoximes with benzoyl chloride in pyridine presumably first gives the corresponding benzoyl-*anti*-aldoxime (not isolated), which is partly decomposed to nitrile and partly isomerized to the benzoyl-*syn*-aldoxime, these two products being isolated. On standing in the reaction mixture the *syn*-derivative is slowly converted to nitrile, presumably through the intermediate formation of the *anti*-derivative.

2. Benzoylation of *anti*-aldoximes in pyridine saturated with hydrogen chloride gives a higher yield of the benzoyl-*syn*-derivative, whereas benzoylation of the *anti*-aldoximes in the presence of triethylamine or tri-*n*-propylamine gives none of the *syn* derivative, the only product being the nitrile.

3. The reaction of *syn*-aldoximes with benzoyl chloride in pyridine gives a high yield of the corresponding benzoyl-*syn*-aldoxime, which, on standing in the reaction mixture, is gradually converted to nitrile.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, NO. 752, AND FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA AT LOS ANGELES]

Magnetic Studies of Ferrihemoglobin Reactions. II. Equilibria and Compounds with Azide Ion, Ammonia, and Ethanol[1]

BY CHARLES D. CORYELL[2] AND FRED STITT[3]

Introduction.—In the first paper of this series dealing with the magnetic properties and structure of ferrihemoglobin (methemoglobin) and some of its compounds,[4] it was shown that the ferric atom is held in the structure with essentially ionic bonds in the acid forms and in the fluoride complex, but that complex formation with hydrosulfide ion or cyanide ion leads to the formation of essentially octahedral covalent bonds between the iron atom and the porphyrin, globin and added group. Complex formation with hydroxide ion (corresponding to an apparent acid-group pK of 8.15 at ionic strength 0.2) leads to magnetic properties intermediate between these two classes, corresponding apparently to the existence of three unpaired electrons or the use of one $3d$ orbital for covalent bond formation.

There are presented in this paper the data which establish the fact that ferrihemoglobin azide contains octahedral covalent bonds to the iron atom. Further studies presented here show that ferrihemoglobin hydroxide forms hitherto unrecognized compounds with ammonia and with ethanol for which equilibrium constants have been determined. Studies of the pH dependence of the ethanol equilibrium indicate that acid ferrihemoglobin also forms ethanol compounds. It is also shown that the magnetic properties of ferrihemoglobin and its hydroxide complex are affected appreciably by methanol and *n*-propanol.

The high sensitivity of the magnetic susceptibilities of ferrihemoglobin and its hydroxide compound to chemical environment, in many cases exceeding the sensitivity of their visual absorption spectra, makes possible quantitative studies of chemical changes which may not directly involve the heme part of the molecule. In the hope of throwing new light on structural relationships in the heme group and between the heme and the protein, globin, we present some of this work from an empirical magnetic standpoint. At some later time, we hope to be able to explain the structural causes of the changes in magnetic susceptibility in the systems containing ammonia and alcohol, and in other systems to be reported in the near future.

Technique of the Measurements.—The various ferrihemoglobin solutions were prepared from cow's blood by the technique described previously[4] involving self-trans-

(1) A portion of this material was presented at the Stanford Meeting of the Pacific Division of the American Association for the Advancement of Science, June, 1939.

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(4) C. D. Coryell, F. Stitt and L. Pauling, THIS JOURNAL, **59**, 633 (1937).

TABLE I

MAGNETIC SUSCEPTIBILITY OF THE IRON IN FERRIHEMOGLOBIN AZIDE

Prepn.	NaN_3, ml.	KOH, ml.	pH	Δw	Corrections: Tube	Corrections: NaN_3	Corrections: KOH	Rw	Dw	Dw_{Hb}	Dw_{COHb}	$10^6\chi_{molal}$
A1	2.00	0	6.3	+2.07	−0.03	+0.07	....	2.11	2.25	10.82	1.04	3,410
A2	2.00	2.50	9.4	1.79	− .03	.07	+0.08	1.91	2.20			3,360
B1	1.11[a]	0	6.7	2.10	− .03	.01	....	2.08	2.16	10.41	1.16	3,520
B2	1.45[a]	0	6.7	1.84	− .03	.02	....	1.83	1.92			3,270
B3	2.41[a]	0	6.7	1.95	− .03	.03	....	1.95	2.10			3,460
C1	0.86	3.26	10.0	1.61	− .03	.03	.11	1.72	1.96	10.64	1.06	3,170
C2	0.86	6.52	11.4	1.81	− .03	.03	.19	2.00	2.50			(3,740)

Mean $10^6\chi_{molal}$ of first six: 3,360; av. dev., 100.

[a] KN_3 solution use.

formation of oxyhemoglobin to ferrihemoglobin on standing two days at room temperature at pH 4.9–5.0, neutralization to pH 6.5–7, and centrifugation to remove the small fraction of denatured protein.

The Gouy apparatus used for determining magnetic susceptibilities also has been described previously.[4] The susceptibilities will be given directly in tables to save space in most cases, but for certain important experiments the full experimental data will be given. The forces in mg. observed with a standard magnetic field (actually an average of the observed forces in two standard fields reduced to one standard) are denoted by Δw. To these are added the $-\Delta w$ of the tube when filled with pure water (blank), and the $-\Delta w$'s experimentally determined for the diamagnetism of reagents that have been added (assuming correction is linear with concentration for dilute solutions), to give the Rw values. Multiplication of Rw by the dilution factor of the original ferrihemoglobin stock solution gives the Dw values, which are hypothetical Δw values that would be obtained with the given ferrihemoglobin solution at its stock concentration in the state of combination being studied but without interference from the diamagnetism of reagents. The values of the molal paramagnetic susceptibility ($10^6\chi_{molal}$) are calculated by use of the equation

$$10^6\chi_{molal} = \frac{(Dw - Dw_{COHb})}{(Dw_{Hb} - Dw_{COHb})} \cdot 12{,}290 \text{ c. g. s. u.} \quad (1)$$

The value of Dw_{COHb} of carbon monoxyhemoglobin corrects for the protein and salt diamagnetism in the stock solution. The equation gives directly the average molal paramagnetism of the iron atom, based on the revised value of that for ferrohemoglobin,[5] providing that all three values of Dw were determined in the same susceptibility tube.

Ferrihemoglobin Azide.—The complex formed between ferrihemoglobin and azide ion involving one mole of each was discovered by Smith and Wolf,[6] and characterized by Keilin[7] with respect to absorption spectrum and stoichiometry. A physico-chemical treatment of Keilin's data indicating the probable participation of heme–heme interaction effects in the equilibrium (leading to a sigmoidal saturation curve similar to those found in ferrohemoglobin equilibria) has been given.[8]

The addition of a 0.474 f potassium azide solution, made up by weighing out the solid, to 30.0 ml. of a ferrihemoglobin solution 0.0147 f in heme iron (at pH 6.7) led to a linear drop of susceptibility with volume of reagent until 0.94 ml. had been added, after which the susceptibility remained constant. The calculated volume of potassium azide solution corresponding to one azide ion per iron atom is 0.93 ml. This magnetic titration provides an excellent check of Keilin's conclusion about the number of azide groups per iron atom, especially since it was made under conditions for which dissociation of the compound is inappreciable.

The determination of the magnetic susceptibility of the iron atom of the complex in the presence of excess azide ion at 25° is presented in Table I. Three different ferrihemoglobin solutions, A, B and C, were used, and potassium hydroxide was added to check the identity of the compound at different pH values and test for occurrence of hydroxide in competition with azide ion. The second column gives the volumes of sodium azide (about 4 f, $\Delta w = -1.19$ when undiluted) or potassium azide (denoted by superscript a, 0.474 f, $\Delta w = -0.37$) solution added to 30.0 ml. of the various ferrihemoglobin solutions. The third column gives the volume of 0.86 N potassium hydroxide solution added ($\Delta w = -1.10$). The eleventh and twelth columns give values of Dw for hemoglobin and carbon monoxyhemoglobin obtained as averages of a number of individual determinations.

The values of $10^6\chi_{mola}$ show satisfactory agreement except for the last value made in very alkaline solution. Because of the probability that the hydroxide ion concentration was high

(5) D. S. Taylor and C. D. Coryell, THIS JOURNAL, **60**, 1177 (1938).
(6) L. Smith and C. G. L. Wolf, *J. Med. Research*, **7**, 451 (1904).
(7) D. Keilin, *Proc. Roy. Soc.* (London), **B121**, 165 (1936).
(8) C. D. Coryell, *J. Phys. Chem.*, **43**, 841 (1939).

enough to prevent complete transformation of the ferrihemoglobin hydroxide complex to the azide complex (competition), we shall neglect this measurement. An experimental study of the quantitative interrelationships in alkaline solutions is in progress.

The average value of susceptibility ($\cdot 10^6$) obtained from the other six determinations, 3,360, corresponds to a magnetic moment of 2.84 Bohr magnetons on the assumption of Curie's law. The expected values for the moments of ferric atoms with one, three, and five unpaired electrons corresponding to the formation of d^2sp^3 octahedral covalent bonds, dsp^2 square covalent bonds, or ionic bonds are, respectively, 1.73 plus orbital moment of 0.3–0.8, 3.87 plus some orbital moment, or 5.92 Bohr magnetons. The value of the moment obtained is consistent with only the first possibility. We conclude that iron atoms of ferrihemoglobin azide form six octahedrally directed covalent bonds, as do those of ferrihemoglobin cyanide and hydrosulfide.[4]

Reduction of the ferrihemoglobin azide solutions at several different *p*H values yields normal ferrohemoglobin, without any magnetic or spectroscopic evidence of complex formation.

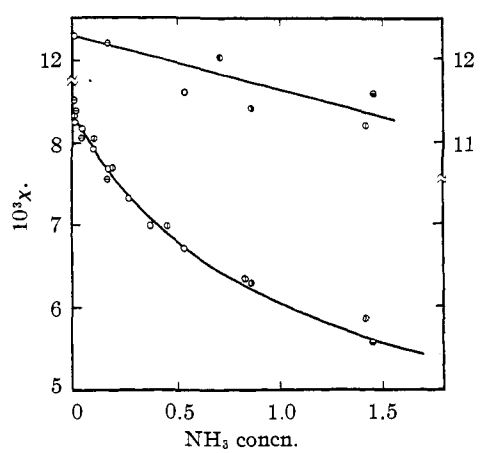

Fig. 1.—Dependence of the paramagnetic susceptibility of ferrihemoglobin hydroxide (lower curve) and of ferrohemoglobin (upper curve) on ammonia concentration; points at *p*H 10.0, ⊖.

Ferrihemoglobin Hydroxide–Ammonia Equilibrium.—It was noted that the magnetic susceptibility of ferrihemoglobin hydroxide solutions (*p*H 10–11) falls considerably with the addition of ammonia solution. No change in spectrum of these solutions was noticed with a hand spectroscope, even when the ammonia concentration was 2 *f*, and the solutions do not undergo precipitation over a period of several days at room temperature.

Several titrations of ferrihemoglobin hydroxide were made with aqueous ammonia solutions at 25°. The results of these are presented in Table II, together with several determinations of the susceptibility of preparations after reduction with

TABLE II

THE MAGNETIC SUSCEPTIBILITIES OF FERRIHEMOGLOBIN HYDROXIDE AND FERROHEMOGLOBIN SOLUTIONS WITH AMMONIA AT *p*H 10.6

Prepn.	Concn. NH_3	$10^6\chi_{molal}$	Prepn.	Concn. NH_3	$10^6\chi_{molal}$
D1[a]	0	8,510	D21	0	8,330
D2⊖	0.0077	8,390	D22	0.098	8,050
D3	.0374	8,050	D23◐	.192	7,700
D4	.162	7,550	D24	.453	6,990
(D4Hb)	.162	12,200)	D25	.830	6,350
			D26	1.42	5,850
D11	0	8,250	(D26Hb	1.42	11,200)
D12	.0396	8,170			
D13	.0977	7,920	D31◑	0.86	6,290[b]
D14○	.173	7,690	(D31Hb	0.86	11,410)
D15	.264	7,320			
D16	.369	6,990	D41◒	1.46	5,600
D17	.533	6,720	(D41Hb	1.46	11,590)
(D17Hb	.533	11,610)	(D51Hb	0.71	12,050)[c]

[a] Initial *p*H 10.0. [b] Δw 5 min. after prepn., 2.84; 30 min., 2.85; 60 min., 2.92; 120 min., 2.99. [c] *p*H 10.6: Δw unchanged after 16 hr.

0.6 g. of sodium dithionite (sodium hydrosulfite, $Na_2S_2O_4$). Successive numbers in the preparation column indicate that the measurements were part of the same titration; the symbol Hb following a number signifies a measurement on a reduced solution. The values in the susceptibility column were obtained in the same manner as the corresponding values in column 13 of Table I. Evidence is presented in the footnotes for preparations D31 and D51Hb that the susceptibilities do not vary rapidly with time for the ferric and ferrous compounds.

The data of Table II are presented in Fig. 1. The ferrihemoglobin hydroxide data in the lower part of Fig. 1 have been treated in accord with the following chemical equation

$$HbOH + NH_3 = HbOH\cdot NH_3 \qquad (2)$$

where total ammonia is treated as NH_3. The curve passing through the points is the theoretical one for this simple chemical equilibrium with the dissociation constant of the complex taken as 1.03, the susceptibility of ferrihemoglobin hydroxide as $8{,}300\cdot 10^{-6}$, and that of the complex as

$3{,}700 \cdot 10^{-6}$. No evidence is observed for the appearance of heme–heme interaction effects on the equilibrium.[8,9]

The points D1–4 measured in less alkaline solutions than the others fall reasonably well on the curve for all the points given in Fig. 1. This fact suggests strongly that the equilibrium constant for reaction 2 is independent of *p*H, and that there is no displacement of the bound hydroxide ion or other change in acidity attendant upon the complex formation. It was attempted to verify this by direct *p*H titration of 25 ml. of ferrihemoglobin as hydroxide complex with suitable mixtures of 4 *f* ammonia and 4 *f* ammonium chloride of the same initial *p*H. These titrations were made by Miss Mary Tobi with a Beckmann *p*H meter. The titration at initial *p*H 10.17 led to a fall to 10.16 followed by a rise to 10.18 for an ammonia concentration of 0.76 *f*. The titration at initial *p*H 10.86 led to a rise to 10.87 followed by a fall to 10.85 for an ammonia concentration of 1.00 *f*.

We conclude that the observed *p*H remained constant within experimental error of ± 0.01 unit. If we assume (A) one equivalent of base is set free per mole of complex formed, the predicted over-all change in *p*H for the first titration is +0.03 plus 0.01, due to decrease in alkali error of the glass electrode (calculated from data supplied by the manufacturer and from sodium and potassium concentrations of the solution), or +0.04; and for the second titration +0.05 (larger due to less buffering power of the ammonia–ammonium system) plus 0.05 or +0.10. If we assume (B) no change in amount of base bound on formation of the complex, the predicted changes are due solely to decrease in alkali error of the electrode or +0.01 and +0.04.

These observations seem to eliminate assumption A, but are not in very good agreement with assumption B, which was suggested by equilibrium data above. In the absence of further information, we shall accept assumption B, that ammonia does not displace base from the molecule. We propose the name ammonia:ferrihemoglobin-hydroxide for the complex, and shall discuss its stereochemistry in a later section of this paper.

The asymptote of the lower curve of Fig. 1 corresponds to the magnetic susceptibility of $3{,}700 \times 10^{-6}$ for the complex. This value is only slightly higher than the value noted for ferrihemoglobin azide, and corresponds to a magnetic moment of 2.93 Bohr magnetons, a moment which we ascribe to one unpaired electron per iron atom.

The data of Table II dealing with ferrohemoglobin susceptibilities measured in the presence of ammonia are presented in the upper part of Fig. 1, above the break in the ordinate scale. A line has been arbitrarily drawn which passes through the well-determined value[5] for zero ammonia concentration, which is unaffected by the high alkalinity prevailing.[9] Part of the spread in values may be attributed to variations in *p*H and therefore in free ammonia due to the acidity of the reducing agent and its oxidation products.

The absorption spectrum of the ammoniacal solution appears to be identical with that of normal ferrohemoglobin. We conclude that ammonia has a specific small effect on the susceptibility of ferrohemoglobin, but we are not able at this time to treat the data quantitatively or to elucidate the nature of the changes occurring.

The Effect of Lower Alcohols on Ferrihemoglobin and Ferrihemoglobin Hydroxide.—Titrations of both ferrihemoglobin at *p*H 6.5 and of ferrihemoglobin hydroxide at *p*H 10.2 were made with 50 volume per cent. solutions of methanol, ethanol, and *n*-propanol. Magnetic effects were noted in all six cases. The first two alcohols did not cause coagulation of very dilute hemoglobin solutions until fairly high concentrations were attained; *n*-propanol caused coagulation at about 15 volume per cent.

None of the alcohols has an appreciable qualitative effect on the absorption spectrum in acid solution, and only ethanol alters appreciably the spectrum of ferrihemoglobin hydroxide. The absorption spectrum observed with large amounts of ethanol is similar to that of ferriheme hydroxide (hematin) but is more sharply defined, consisting of a broad band about 300 Å. wide with a maximum at about 5980 Å. and a band including practically all wave lengths shorter than 5300 Å. (Normal ferrihemoglobin hydroxide has an overlapping two banded spectrum with maxima in absorption at 5800 Å. and 5475 Å.)

The general observations of the magnetic susceptibilities of ferrihemoglobin and ferrihemoglobin hydroxide as a function of the alcohol concentration with the three alcohols are presented in Fig. 2. The various measurements are presented as various kinds of solid-line circles, with arbitrary solid-line curves. In acid solu-

(9) F. Stitt and C. D. Coryell, THIS JOURNAL, **61**, 1263 (1939).

tion methanol causes a very small drop in susceptibility, ethanol a noticeable rise, and propanol a pronounced fall accompanied by coagulation of the (relatively concentrated) ferrihemoglobin solution (20%) at 6 volume per cent. and gel formation at 9.7%. In alkaline solution methanol also causes a small drop in the susceptibility from the initial value, ethanol a very pronounced rise on a curve which later flattens out, and propanol a rise with partial precipitation even at the low concentrations studied.

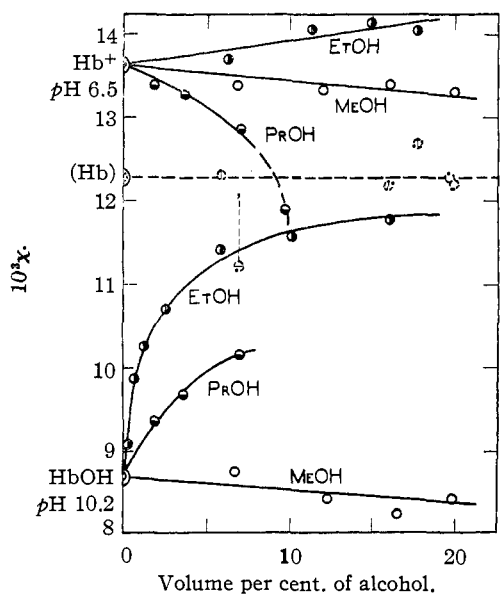

Fig. 2.—The effect of some alcohols on the paramagnetic susceptibility of ferrihemoglobin at *p*H 6.5 (upper curves), ferrihemoglobin hydroxide at *p*H 10.2 (lower curves), and ferrohemoglobin (broken line).

Reduction of the alcoholic ferrihemoglobin solutions by sodium dithionite, even in the presence of considerable methanol or ethanol, leads to the susceptibility value and absorption spectrum characteristic of normal ferrohemoglobin. The susceptibilities obtained for the reduced solutions are indicated in Fig. 2 by broken circles. The value for normal ferrohemoglobin is represented by the horizontal broken line. The high value obtained with 17.9 volume per cent. ethanol in acid solution is in error, since other similar preparations gave the normal value on reduction. The low value obtained by reduction of the *n*-propanol solution at 6.7 volume per cent. was obtained in the presence of coagulum, but no hemochromogen absorption bands were observed.

We believe that the deviations in susceptibilities from the normal values which are observed in the different alcoholic solutions are significant. The denaturation and precipitation caused by *n*-propanol make the effects difficult to study with the magnetic method, which requires concentrated hemoglobin solutions. The effects observed with methanol are too small for accurate magnetic study, but they may be due to changes in the protein molecule which give rise to second-order effects at the iron atom. The reaction with ethanol has been studied in detail and is discussed in the following sections.

The Ethanol:Ferrihemoglobin-Hydroxide System and Complexes Formed.—The curve for the magnetic susceptibility of ferrihemoglobin hydroxide in alkaline solution as a function of the ethanol concentration (Fig. 2) corresponds to the shape expected for the formation, as ethanol is added, of an increasing quantity of a complex more paramagnetic than ferrihemoglobin hydroxide. Since there also is presumptive evidence for complex formation in acid solution, experiments were carried out to determine the apparent pK value of the constant (pK_3)[10] corresponding to the following possible reaction, in which it is assumed that there is one ethanol molecule per iron atom in each form of the complex

$$Hb^+\cdot EtOH + OH^- = HbOH\cdot EtOH \qquad (3)$$

The dissociation constant $K_{diss.}$ of the hydroxide ethanol complex into the acid ethanol complex and hydroxide ion is related to pK_3 and the ionization constant of water K_w by the equation

$$\log K_{diss.} = pK_3 + \log K_w \qquad (4)$$

In almost any case it would seem probable that combination with ethanol would affect the dissociation of the iron-hydroxide complex. The magne ic–*p*H titrations described below were undertaken to determine the magnitude of the effect of ethanol on the value of pK_3.

The results of the titrations of ferrihemoglobin at 25° with potassium hydroxide in the presence of 9–10 and 18–20 volume per cent. ethanol and of a back-titration of ferrihemoglobin hydroxide with lactic acid (HLa) in 9–10% ethanol are presented in Tables III-A and III-B. The Δw of 10% ethanol was found to be +0.48, and of 20% ethanol, +0.96, of 0.87 N potassium hydroxide, −1.11, of 1 N lactic acid, 0.00, and the values of Dw_{Hb} and Dw_{COHb} for the ferrihemo-

(10) We designate this group with subscript 3 since two other pK's associated with the heme are known for ferrihemoglobin. The first of these has in normal ferrihemoglobin the value 5.3; the second has the value 6.65, but is not detected magnetically. See the paper by C. D. Coryell and L. Pauling, *J. Biol. Chem.*, **132**, 769 (1940).

TABLE III-A

MAGNETIC TITRATIONS OF FERRIHEMOGLOBIN SOLUTIONS WITH HYDROXIDE ION IN 9–10% ETHANOL AT 25°

Point	Vol. Hb^+	Vol. 40% EtOH	Vol. KOH	Vol. HLa.	Vol.-% EtOH	pH	Δw	Corrections: Tube	Corrections: EtOH	Corrections: KOH	Rw	Dw	$10^6\chi_{molal}$
E-1	24.0	8.0	0	0	10.0	5.95	8.22	0.85	−0.48	..	8.59	11.45	13,910
E-2			0.90		9.7	6.90	8.02	.85	− .47	0.03	8.43	11.53	14,000
E-3			1.80		9.5	7.71	7.54	.85	− .46	.05	7.98	11.23	13,660
E-4			2.70		9.2	8.91	6.11	.85	− .44	.08	6.60	9.54	11,730
E-5			3.60		9.0	9.79	5.75	.85	− .43	.11	6.28	9.32	11,500
E-6			5.00		8.7	10.45	5.27	.85	− .42	.15	5.75	8.86	10,990
E-11	28.0	7.0[a]	0.90[b]	0	10.6	10.29	7.10	.85	− .52	.19	7.62	9.77	12,010
E-15	25.0	10.0	4.50	0	10.1	10.2	5.75	1.05	− .49	.13	5.94	9.38	11,580
E-21	24.2	8.5	0.80[b]	0	10.1	10.40	6.78	0.03[c]	− .49	.17	6.49[c]	9.11	11,270
E-22				1.00	9.9	9.90	6.63	.03[c]	− .48	.16	6.34[c]	9.16	11,320
E-23				2.00	9.6	8.78	6.76	.03[c]	− .46	.16	6.49[c]	9.65	11,880
E-24				2.40	9.5	8.22	7.17	.03[c]	− .46	.16	6.90[c]	10.38	12,710
E-25				2.90	9.4	7.65	7.43	.03[c]	− .45	.16	7.17[c]	10.91	13,300
E-26				3.40	9.2	7.22	7.50	.03[c]	− .44	.15	7.24[c]	11.18	13,600

TABLE III-B

MAGNETIC TITRATIONS IN 18–20% ETHANOL AT 25°

E-31	12.5	12.5	0	0	20.0	6.09	6.00	0.85	−0.96	..	5.89	11.78	14,310
E-32			0.30		19.8	6.71	5.90	.85	− .95	0.01	5.81	11.76	14,290
E-33			.60		19.5	7.24	5.75	.85	− .94	.02	5.68	11.64	14,130
E-34			.90		19.3	7.78	5.51	.85	− .93	.04	5.47	11.33	13,780
E-35			1.20		19.1	8.43	4.93	.85	− .92	.05	4.91	10.29	12,630
E-36			1.50		18.9	9.23	4.76	.85	− .91	.06	4.76	10.10	12,400
E-37			1.80		18.7	9.79	4.52	.85	− .90	.07	4.54	9.74	11,990
E-38			2.10		18.4	10.08	4.45	.85	− .88	.08	4.50	9.76	12,010

[a] 4.5 ml. of 40% plus 2.5 ml. of 80% EtOH. [b] 5.4 *N* KOH. [c] Different tube used; multiply *Rw* by 1.013 for cross-section correction before applying dilution correction.

globin solution D used were determined as +10.00 and −0.85. It was deemed unnecessary to correct *p*H values for the alkali ion error, or for the small effect of the ethanol on the *p*H reading.

The data of Tables III-A and III-B were treated independently by the method described previously[4] to determine the best values of the asymptotic susceptibilities χ_{Hb^+} and χ_{HbOH} ($\cdot 10^6$) in acid and alkaline solutions and of the pK_3 corresponding. The data of Table III-A yielded χ_{Hb^+} = 14,000, χ_{HbOH} = 11,400, pK_3 = 8.19; those of Table III-B yielded χ_{Hb^+} = 14,300, χ_{HbOH} = 12,000, pK_3 = 8.23. The reliability of the χ values[11] is approximately ±100, that of the pK_3 values, approximately ±0.10. For comparison, we present the values for normal ferrihemoglobin: χ_{Hb^+} = 13,910, χ_{HbOH} = 8,250, and pK_3 = 8.15 at ionic strength 0.2.

The data of Tables III-A and III-B are presented graphically in Fig. 3, together with the theoretical curves A and B based on the asymptotic susceptibilities given above and on the rounded-off value of pK_3 of 8.20 for each curve.

(11) These values refer to averages for the various forms of ferric atoms in the equilibrium at the given alcohol concentration.

Point E-11 (in 10.6% ethanol) lies unaccountably high, but on the whole the curves represent the data very satisfactorily. Data were not obtained at low enough *p*H for the determination of the relationships involving the most acid of the heme-linked acid groups.[10] We conclude from the derivation of the parameters and from

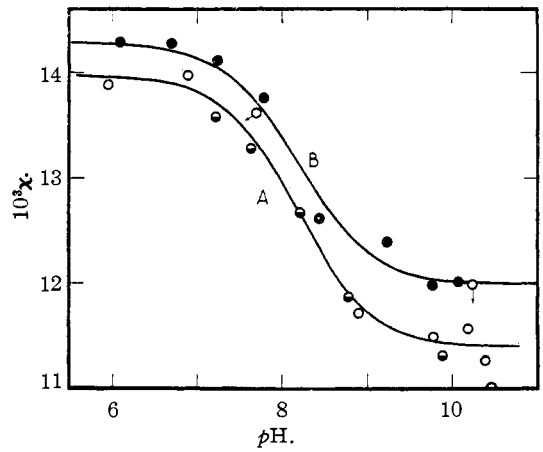

Fig. 3.—Dependence of paramagnetic susceptibility of alcoholic ferrihemoglobin solutions on *p*H: Curve A, 9–10% EtOH, KOH titration ○, HLa titration ◒; Curve B, 18–20% EtOH, KOH titration ●.

the fit of the two curves that pK_3 does not have an appreciable dependence on ethanol concentration. The significance of this will be discussed in the next section.

It is also concluded that the ethanol equilibrium with ferrihemoglobin hydroxide can be studied equally well magnetically at any point in the pH range 9.5–11, since the susceptibilities are independent of pH in this range. A number of experiments were made to determine the susceptibility of solutions as a function of the ethanol concentration (for which purpose the volume molality was taken as 0.176 times the volume per cent.). It was found that the blank determinations of the susceptibility of ferrihemoglobin hydroxide did not always lead to the same value, possibly due to denaturation during the addition of potassium hydroxide (0.87 or 5.3 N) and to differences in concentration of hemoglobin-like impurities[5] for the different preparations, one of which (F) was quite old. Because of this unexpected difficulty, the value of the susceptibility observed in the absence of alcohol at the start of a titration was subtracted from the values at various alcohol concentrations to give values of the increase in susceptibility $\Delta\chi$ caused by the ethanol, values which then showed satisfactory experimental reproducibility.

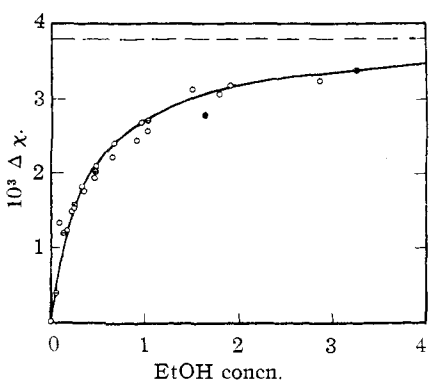

Fig. 4.—Increase in magnetic susceptibility ($10^3\Delta\chi$) of ferrihemoglobin hydroxide caused by complex-formation with ethanol. Broken curve is asymptote.

The results of four titrations at 25° of ferrihemoglobin hydroxide solutions E and F of initial pH 9.8 to 10.6 with ethanol are presented in Table IV and in Fig. 4. From the susceptibilities given in the third column are subtracted the susceptibilities observed for the first points of the titrations measured at zero alcohol concentration. The alkaline asymptotes of curves A and B of Fig. 3 have also been used as a source of information in connection with an average experimental value for normal ferrihemoglobin hydroxide of solution E.

TABLE IV

THE INCREASE IN MAGNETIC SUSCEPTIBILITY OF FERRIHEMOGLOBIN HYDROXIDE IN ETHANOL SOLUTIONS

Point	EtOH, molal	$10^6\chi_{molal}$ obsd.	$10^6\Delta\chi$
E-50	0	8,830	..
E-51	0.122	10,020	1,190
E-52	.240	10,360	1,530
E-53	.348	10,590	1,760
E-54	.459	10,770	1,940
E-55	.662	11,240	2,410
E-56	.950	11,510	2,680
E-57	1.89	12,010	3,180
E-60	0	8,480	..
E-61	0.162	9,790	1,310
E-62	.326	10,300	1,820
E-63	.477	10,570	2,090
E-64	.905	10,930	2,450
E-65	1.50	11,610	3,130
E-70	0	8,520	..
E-71	0.230	10,010	1,490
E-72	.650	10,740	2,220
E-73	1.02	11,100	2,580
E-74	1.79	11,580	3,060
E-75	2.85	11,770	3,250
F-0	0	8,690	..
F-1	0.060	9,080	390
F-2	.108	9,860	1,170
F-3	.231	10,250	1,560
F-4	.448	10,710	2,020
F-5	1.02	11,410	2,720
Asymptotes			
Fig. 3, A	1.61	11,400	2,790[a]
Fig. 3, B	3.26	12,000	3,390[a]

[a] Based on the average of points E-50, 60, 70 for normal ferrihemoglobin hydroxide.

We have been able to fit a theoretical curve to the data of Table IV, which are given in Fig. 4. The curve is based upon the chemical equation

$$\mathrm{HbOH} + \mathrm{EtOH} = \mathrm{HbOH{\cdot}EtOH} \qquad (5)$$

with asymptotic $10^6\Delta\chi$ (the increase in molal susceptibility of the iron atom on forming the ethanol complex) as 3,800 and K (the dissociation constant of the complex to ferrihemoglobin hydroxide and ethanol) as 0.39. No evidence is observed in these data for the role of heme–heme interactions leading to sigmoid saturation curves.[8]

Since the data of Tables III-A and III-B bring us to the conclusion that pK_3 for the ferrihemoglobin–ferrihemoglobin hydroxide transition is independent of the concentration of ethanol up to 19 volume per cent. ethanol (where 83% of the

TABLE V

CORRECTED MOLAL PARAMAGNETIC SUSCEPTIBILITIES, EFFECTIVE MAGNETIC MOMENTS, AND THE DISSOCIATION CONSTANTS OF SOME FERRIHEMOGLOBIN COMPOUNDS (AT 25°)

Compounds	Symbol	$10\chi^{g}_{molal}$	$\mu_{eff.}$	$K_{diss.}$
Ferrihemoglobin (Form I)	H_2Hb^+	12,430	5.46	$5.0\cdot10^{-6}$
Ferrihemoglobin (Forms II, III)	HHb^+, Hb^+	13,910	5.77	
Ethanol: ferrihemoglobin	$[Hb^+]EtOH$	14,500	5.89	0.4
Ferrihemoglobin azide	HbN_3	3,360	2.84	$1.2\cdot10^{-5}$ [a]
Ferrihemoglobin hydroxide	HbOH	8,250	4.45	
		(8,630)[b]	(4.55)[b]	$1.3\cdot10^{-6}$ [c]
Ammonia: ferrihemoglobin-hydroxide	$[HbOH]NH_3$	3,700	2.98	1.0
Ethanol: ferrihemoglobin-hydroxide	[HbOH]EtOH	12,150	5.39	0.39
		(12,530)[b]	(5.48)[b]	

[a] Azide concentration at half saturation; saturation curve is sigmoidal, preventing direct comparison of dissociation constants with the others observed for simple equilibrium.[8] [b] Values found in this investigation with preparations E and F. [c] Ionic strength of 0.1, $K_{diss.}$ defined by eqn. (4).

ferrihemoglobin hydroxide is in the ethanol complex), we conclude that the dissociation constant of the (acid) ethanol:ferrihemoglobin complex to ethanol and ferrihemoglobin is within experimental error the same as that of the hydroxide–ethanol complex or approximately 0.4. The upper ethanol curve of Fig. 2 should therefore be of the same form as the lower one with an asymptote of about $14,200 \times 10^{-6}$ for the susceptibility of the complex under these conditions or an increase of about 600×10^{-6} in susceptibility upon complex formation.

Discussion.—The molal paramagnetic susceptibilities of the iron, and dissociation constants of the ferrihemoglobin compounds studied in this investigation and of the acid and basic forms of ferrihemoglobin itself are collected in Table V. Provisional symbols corresponding to the names and indicating the mode of dissociation referred to in the calculation of the constants are given in the second column. The values of the effective magnetic moments $\mu_{eff.}$, assuming Curie's law and independence of hemes, are given in column four.

The magnetic susceptibility ($\cdot10^6$) of ferrihemoglobin in its most acid form I (H_2Hb^+) has been reported[10] to be 12,570. The increase in susceptibility on losing a proton ($pK_1 = 5.3$) is 1,500, leading to a value of 14,070 for form II (HHb^+). Both of these values must be lowered by 1.14% to correspond to the new ferrohemoglobin standard value of 12,290, since the values are based on that for ferrohemoglobin by virtue of eqn. (1); this correction leads to new values of 12,430 and 13,910. These values are average values for the iron in the ferrihemoglobin preparations, and cannot as yet be corrected for the effect of hemoglobin-like impurities[5] (4–8%) which are present in the freshly drawn blood and seem to be ferric compounds of somewhat low paramagnetism capable of combining with carbon monoxide after reduction. The loss of a second proton to give form III (Hb^+) does not make a detectable difference in the magnetic susceptibility.

The previously reported value[4] for ferrihemoglobin hydroxide is 8,340 based on studies of three hemoglobin preparations, which becomes 8,250 on correction for the new ferrohemoglobin value. Although the average susceptibility value found (points 1, 11, and 21 of Table II) for preparation D (8,360) is in satisfactory agreement with the corrected value, the average value for preparation E (points 50, 60, and 70 of Table IV) is 8,610, and the value found for preparation F in an extensive study is $8,650 \pm 80$,[12] corrected to the new standard. It is possible that the observed paramagnetism of this complex is very sensitive to the amount of the abnormal hemoglobin impurity found in the blood source. We are unable to offer any other explanation for the differences in the values found for the susceptibility of this compound.

The susceptibility of the ethanol:ferrihemoglobin-hydroxide complex is 3,800 units higher than that of ferrihemoglobin hydroxide itself. We leave for the present the exact value for these hydroxide complexes open to further study, recording both extreme values found. The value for the acid form of the ethanol complex is taken as 600 units higher than that of ferrihemoglobin form II or III.

(12) The corresponding value for susceptibility of the acid forms II and III was found to be lower by 240 units (av. dev. ±70); while pK_3 was found to be 8.07 at ionic strength 0.18.

The addition of ethanol to either the acid or the alkaline form of ferrihemoglobin leads to a rise in magnetic moment. In the acid complex the moment approaches closely to the theoretical value (5.92) predicted for a ferric atom with five unpaired electrons (^6S) forming no covalent bonds involving $3d$ orbitals. This high value has also been observed for ferrihemoglobin fluoride[4] and for many simpler ferric complexes of essentially ionic nature.

The value observed for ethanol ferrihemoglobin hydroxide is higher than that observed for any other ferrihemoglobin compound except the two mentioned above. The value is much higher than that expected for a ferric atom with one $3d$ orbital being used in bond formation, having three unpaired electrons, a class to which ferrihemoglobin hydroxide may belong.[4] Since the normal state of a ferric ion is ^6S with no orbital contribution to the paramagnetism predicted, it is difficult to interpret values which are high but which fall definitely short of 5.92 unless a mixture of forms be present at room temperature, the equilibrium being shifted in complex formation. We recognize, however, that the occurrence of high susceptibility and moment is characteristic qualitatively of the predominant ionic character of the complex.

A surprising feature of the formation of complexes between ferrihemoglobin hydroxide and ammonia or ethanol is the evidence that the complex formers add to the heme groups without evidence that the hydroxide ion is displaced. Since the formation of either of these complexes involves a large change in the paramagnetism of the iron atom, we conclude that direct bonding to the iron atom (covalent for ammonia and ionic for ethanol) has probably occurred. A difficulty is that the iron atom in normal ferrihemoglobin hydroxide has until now been considered effectively space-saturated in the octahedral bonding of six groups, namely, the hydroxide ion, the four porphyrin nitrogen atoms and one imidazole nitrogen atom from a histidine residue of the globin.[10]

Professor Pauling has suggested to us that the complex-forming molecule may actually displace the hydroxide ion, and at the same time there may occur a shift in the ionization constant of some hitherto unrecognized heme-linked acid group from a higher value down to a value less than pK 9.5 for the ammonia complex, and to pK 8.2 for the ethanol complex, simulating accidentally in the latter case the observed pK_3 for addition of the hydroxide ion to ordinary ferrihemoglobin. If this is the case, this acid group must also be closely connected with the porphyrin-iron structure to have its ionization bring about a large change in susceptibility in the ethanol system.

A second possibility is that the ethanol or ammonia molecule may displace the imidazole nitrogen of the globin-iron link (leaving the heme group attached to the protein elsewhere, as by the two propionate side chains). This latter mechanism of addition would probably involve a change in the other pK values of heme-linked acid groups,[10] but we have information available only on the pK_3 of the ethanol system, which has not been altered appreciably, perhaps because of cancellation of effects.

There is also the possibility that hepta-coördination of ferric iron can occur. Evidence is accumulating that the iron atom of protein-free porphyrin complexes may bind three groups in addition to the quadridentate coplanar porphyrin addendum.[13] Two structures have been proposed for hepta-coördination: the introduction of the seventh bond in a face of an octahedron with subsequent adjustment to spread bonds somewhat,[14] and the bifurcation of one bond of an octahedron with subsequent spreading of bonds.[15] The latter seems to be a possibility for the explanation of the structure of these hemoglobin complexes, with bifurcation occurring with the iron bond opposite the protein leaving the equatorial bonds to the porphyrin molecule largely unchanged.

If this is the case with the ethanol:ferrihemoglobin-hydroxide complex with ionic bonds, it is also probable that the acid form has a similar structure with the ethanol molecule and a water molecule held in the same manner, with no effect of the ethanol on the acidity of the aquo group. If this is the case with the ammonia:ferrihemoglobin-hydroxide complex, the bonds may be d^3sp^3 with an unpaired electron in the $5d$ orbital, or may involve essentially covalent d^2sp^3 hexacoördination with resonance of the six covalent

(13) T. H. Davies, *J. Biol. Chem.*, **135**, 597 (1940); W. M. Clark and M. E. Perkins, *ibid.*, **135**, 643 (1940).

(14) Proposed by G. C. Hampson and L. Pauling, THIS JOURNAL, **60**, 2702 (1938), to explain the structure of the ZrF_7^{---} complex in K_3ZrF_7 and $(NH_4)_3ZrF_7$.

(15) Found by J. L. Hoard, *ibid.*, **61**, 1252 (1939), for the complexes CbF_7^{--} and TaF_7^{--} in their potassium and ammonium salts.

bonds among seven positions, with possible use of a $5d$ orbital for covalent character of the seventh bond.

All three of these explanations involve the assumption of fortuitous changes or cancellations of changes to explain the observation that pK_3 has the same value in the presence and absence of ethanol. It will be highly desirable to obtain more evidence from other compounds to clarify the structure of these complexes.

It is worth noting that these complexes reported and the one between ferrihemoglobin and imidazole reported by Russell and Pauling[16] are the only ones known to date between ferrihemoglobin and a neutral electron donor. It is probable that ionization constants of heme-linked acid groups[10] will differ between this class of electron donors and the anion classes including azide, cyanide, fluoride, fulminate,[17] hydrosulfide, and hydroxide ions, already known.

We are grateful to Professor Linus Pauling and to Dr. T. Harrison Davies for stimulating advice and assistance in this work.

Summary

The effects of azide ion, ammonia, methanol, ethanol, and *n*-propanol on the magnetic properties of ferrihemoglobin (methemoglobin) solutions have been studied at 25°. The magnetic properties of ferrihemoglobin azide have been established. It is found that ammonia forms a complex with ferrihemoglobin hydroxide and the dissociation constant of this complex, tentatively identified as ammonia:ferrihemoglobin-hydroxide, has been measured. Ethanol is found to form a complex with ferrihemoglobin and another with ferrihemoglobin-hydroxide. The dissociation constant of the latter (ethanol:ferrihemoglobin-hydroxide) is found to be 0.39; the equilibrium between the two complexes and hydroxide ion is found to have the same constant as the corresponding equilibrium between ferrihemoglobin and ferrihemoglobin-hydroxide, whence the dissociation constant of ethanol:ferrihemoglobin is found to be 0.4. Propanol has a pronounced effect on magnetic properties but leads to denaturation. Methanol has a slight influence on magnetic properties.

The paramagnetic part of the molal susceptibilities correspond to the following effective magnetic moments per heme, in Bohr magnetons, ferrihemoglobin azide, 2.84; ammonia:ferrihemoglobin-hydroxide, 2.95; ethanol:ferrihemoglobin, 5.89; ethanol:ferrihemoglobin-hydroxide, 5.39. For the azide and ammonia compounds these correspond to one unpaired electron per iron atom, indicating essentially covalent bonds; for the ethanol compounds, to five unpaired electrons per iron atom, indicating essentially ionic bonds.

PASADENA, CALIF.
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(16) C. D. Russell and L. Pauling, *Proc. Nat. Acad. Sci.*, **25**, 517 (1939).

(17) R. D. Barnard and W. Neitzel, *Proc. Soc. Exptl. Biol. Med.*, **39**, 462 (1938).

[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

Interaction of Ions and Dipolar Ions. I. Solubility of Barium and Calcium Iodates in Glycine and in Alanine Solutions

BY R. M. KEEFER, H. G. REIBER AND C. S. BISSON[1]

It has been shown by many workers that the aliphatic alpha amino acids exist in aqueous solution as dipolar ions. In investigations involving dipolar ion solutions it is often desirable to know the activity of the simple ions present. By extending the Debye–Hückel theory to the case where one of the ions is a dipolar ion, Kirkwood[2] has obtained a limiting law for the interaction of ions and dipolar ions. Furthermore, he has shown that the solubility of glycine[2] in alcoholic solutions containing salts is in agreement with the limiting law. Electrode studies of Joseph[3] and solubility measurements of Failey[4] involving salts in aqueous amino acid solutions, after a "salting out" effect[5] correction, are also in agreement with the Kirkwood limiting law. As Failey's[4] solubility measurements were with 1–1 type salts, the present investigation gives the results of solubility

(1) This paper is being published following the death of Prof. C. S. Bisson, and responsibility for all statements will be assumed by his collaborators.

(2) Kirkwood, *J. Chem. Phys.*, **2**, 351 (1934).

(3) Joseph, *J. Biol. Chem.*, **111**, 479 and 489 (1935); **130**, 203 (1939).

(4) Failey, THIS JOURNAL, **55**, 4374 (1933).

(5) Kirkwood, *Chem. Rev.*, **24**, 233 (1939).